

**TRACER REAGENTS THAT ENHANCE REACTION-PRODUCT ANALYSIS**

REFERENCE TO RELATED APPLICATIONS

This application is a non-provisional application  
claiming priority from provisional application serial no.  
5 60/143,009 filed July 9, 1999.

BACKGROUND OF THE INVENTION

The present invention generally relates to reagents  
that are essential for an enzymatic reaction and that  
enhance reaction-product analysis. In specific,  
10 preferred embodiments, the invention provides  
compositions of essential components which facilitate  
subsequent chromatographic or electrophoretic analysis.

Enzymes are frequently used in laboratories to  
catalyze a variety of transformations. Typical enzymes  
15 which have been utilized include proteases, peroxidases,  
oxidases, kinases, amylases, and several nucleic acid  
modifying enzymes such as DNA polymerases, RNA  
polymerases, ligases, kinases, restriction endonucleases,  
phosphodiesterases, DNases, exonucleases, RNases, and  
20 phosphatases. The nucleic acid-modifying enzymes have  
been frequently used in molecular biology laboratories as  
part of procedures such as polymerase chain reaction  
("PCR"), sequencing, southern hybridization analysis,  
restriction endonuclease analysis, RNase protection, and  
25 the production of labeled probes.

The steps involved in performing an enzyme catalyzed  
transformation can generally be categorized as reaction  
mixture formulation, enzymatic reaction, reaction product  
characterization, and reaction product use. The steps of  
30 mixture formulation, product characterization, and  
product use are labor intensive. The formulation of  
enzyme reaction mixtures entails combining reaction  
components which are essential for the enzymatic reaction

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into a reaction mixture. The reaction mixture is then incubated under conditions favorable for the enzymatic reaction to take place, and for a time sufficient to allow the enzymatic reaction to proceed substantially to completion. The reaction mixture is typically analyzed to evaluate the characteristics of the products formed. This analysis often entails a chromatographic or electrophoretic procedure to separate and evaluate the reaction products, and to determine whether the enzymatic reaction has proceeded to completion. Downstream applications entail a wide variety of varied uses for the products of enzymatic reactions, such as utilization in manufacturing, and further processing of the product with enzymes or chemical processes. In the case of molecular biological enzymatic reactions, examples of downstream applications are transformation of prokaryotic or eukaryotic cells, detection of complementary sequences by southern or northern hybridization, sequencing, phosphorylation, dephosphorylation, ligation, restriction digestion, endonucleolytic digestion, exonucleolytic digestion, and purification.

Procedures such as liquid chromatography and polyacrylamide gel electrophoresis ("PAGE") have been frequently used to analyze the results of the enzymatic reactions by separating the reaction products by, for example, molecular weight. The results of the modification of nucleic acids by enzymes such as DNA polymerase have typically been analyzed by subjecting the reaction products to electrophoresis through polyacrylamide or agarose gels.

To analyze enzymatic reaction products using chromatography or electrophoresis, the sample to be analyzed has often been combined with components which assist the operator in performing the separation. One such component is a "tracer", which is a detectable

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moiety such as a dye which is generally added to the sample immediately before loading the sample onto the chromatography column or electrophoresis gel. The tracer migrates in the medium in the same direction as the sample to indicate the progress of the separation.

Another reagent, termed "high density agent" herein, has also been commonly utilized in electrophoretic analysis of the enzymatic reaction products. High density agents are generally water soluble, dense liquids, such as a solution of sucrose or glycerol, which have been mixed with the sample, usually after the enzymatic reaction is complete, to increase the sample density. The increased density of the sample resulting from mixing the sample with the high density agent aids, for example, in loading the sample into a well of an electrophoresis gel by allowing the sample, when pipetted into the top of the well, to "fall" through the less-dense electrophoresis buffer solution to the bottom of the well.

High density agent and tracer have been combined with reaction product-containing samples to be electrophoretically separated. The combination of high density agent and tracer is generally termed "loading buffer".

While tracers and loading buffers have usually been mixed with the sample after the enzymatic reaction is complete, as diagramed in Fig. 1, their use before the commencement of nucleic acid-modifying enzymatic reactions is also known, as diagramed in Fig. 2. Hoppe et al., BioTechniques 12:679-680 (1992) describe combining a solution of sucrose (up to 30%) and certain dyes (cresol red, tartrazine, or yellow food coloring #5) with an enzyme reaction mixture containing all other components for PCR. After the PCR procedure, the samples were reportedly loaded directly onto an agarose gel for

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electrophoretic analysis. The authors noted that several dyes and heavy components were inhibitory to the Taq polymerase enzyme used, but that sucrose, cresol red, tartrazine, and yellow food coloring #5 were compatible  
5 with Taq.

Certain commercially available products provide tracer or loading buffer for use in enzyme reaction mixes for PCR. A thermostable polymerase preparation, Red Hot DNA Polymerase, is available from Advanced  
10 Biotechnologies and reportedly contains a red dye for use to indicate enzyme addition in the enzyme reaction mixture. There are also two products available which comprise a red tracer and a high density agent, for addition to a PCR reaction mixture before amplification.  
15 One, called RediLoad, is available from Research Genetics, Inc., and the other, called Rapid-Load™, is available from OriGene Technologies. These reagents must be added to the reaction mixture in a separate pipetting step.

20 SUMMARY OF THE INVENTION

Despite the efforts and improvements made in the prior art, inefficiencies in reaction mixture formulation and reaction product analysis still exist. In particular, there is no product to date which combines an  
25 essential component for an enzyme reaction with a tracer and/or high density agent which can be used in an enzymatic reaction and provide sufficient tracer and/or high density agent such that the product of the enzyme reaction could be directly evaluated in a chromatographic  
30 or electrophoretic procedure without supplying additional tracer or high density agent. Such a product ("analysis reagent composition") would provide additional advantages over the products currently available because it would  
(1) indicate reagent addition into the enzymatic reaction

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mix, and (2) eliminate the need for separately adding a loading buffer since the loading buffer components are added along with the essential reagent.

Among the several objects of the invention,  
5 therefore, is the provision of compositions for use in formulating enzymatic reaction mixtures that offer improved efficiencies in connection with the labor intensive protocols for reaction mixture formulation, and reaction product characterization.

10 The invention is thus generally directed to the provision of a composition comprising an essential component of an enzymatic reaction combined with a tracer which is compatible with the enzyme, where the composition contains an essential absence of the  
15 substrate. The composition can have a density at least about 1.01 g/cm<sup>3</sup>. This composition is particularly useful for any enzyme reaction where post-reaction processing or analysis is benefited by the tracer and/or increased density of the reaction mixture. In particular, such  
20 compositions for polymerase and restriction enzyme reactions are provided, where the presence of the tracer and/or increased density is useful for post-reaction electrophoretic analysis. Methods for using these compositions, and methods for preparing these  
25 compositions are also provided.

The invention is directed, therefore, to a composition which is suitable for formulation of an enzymatic reaction mixture, the composition comprising a reaction component essential for an ex-vivo non-  
30 polymerase enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer compatible with the enzyme, wherein the composition is substantially free, or has an essential absence, of the substrate. These compositions

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can further comprise a density of at least about 1.01 g/cm<sup>3</sup>.

The present invention is also directed toward a composition comprising a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product and a tracer compatible with the enzyme, the composition being substantially free or having an essential absence of the substrate and having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance.

The present invention is further directed toward a composition comprising a reaction component essential for an ex-vivo polymerase reaction in which a nucleic acid polymer product complementary to a nucleic acid polymer template is prepared, and a tracer compatible with the polymerase, the composition being substantially free or having an essential absence of the template and has an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance. These compositions can also comprise a density of at least about 1.01 g/cm<sup>3</sup>.

The present invention is still further directed toward a composition for an enzymatic reaction component which comprises a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and an alkaline earth-metal salt of an anionic tracer.

The present invention is also directed toward a composition which comprises a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer selected from the group consisting of acid red 106, acid red 4, acid red 1, amaranth, and acid violet 5, or a salt thereof.

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The present invention is further directed toward a composition comprising a reaction component essential for an ex-vivo enzymatic reaction in which a nucleic acid polymer substrate is enzymatically cleaved by a

5 restriction enzyme in a reaction mixture to form a restriction product, and a tracer compatible with the restriction enzyme, wherein the composition contains an essential absence of the substrate. These compositions can also comprise a density of at least about 1.01 g/cm<sup>3</sup>.

10 The present invention is still further directed toward an improvement in a method for a polymerase reaction that comprises forming a reaction mixture comprising a polymerase, a nucleic acid polymer template, a tracer compatible with the polymerase, and other  
15 components essential for the polymerase reaction, creating a nucleic acid polymer product complementary to the nucleic acid by enzymatic reaction, analyzing the product of the enzymatic reaction by an electrophoretic protocol, and observing the tracer during the  
20 electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture. The improvement comprises supplying the tracer to the reaction mixture in a composition that comprises the tracer and the enzyme or another essential component,  
25 the composition being substantially free or having an essential absence of the nucleic acid polymer template. A further improvement is in the reaction mixture having a density at least about 1.01 g/cm<sup>3</sup>.

The present invention is further directed toward an  
30 improvement in a method for a polymerase reaction that comprises forming a reaction mixture comprising a polymerase, a nucleic acid polymer template, a tracer compatible with the polymerase, and other components essential for the polymerase reaction, creating a nucleic  
35 acid polymer product complementary to the nucleic acid by

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enzymatic reaction, analyzing the product of the enzymatic reaction by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol. The improvement comprises supplying the tracer to the  
5 reaction mixture in a composition that comprises the tracer and the enzyme or another essential component, the composition being substantially free or having an essential absence of the nucleic acid polymer template, wherein the tracer supplied to the reaction mixture is of  
10 adequate character and sufficient quantity to be visible during the electrophoretic protocol.

The present invention is also directed toward a method for a restriction enzyme reaction, the method comprising forming a reaction mixture comprising a  
15 restriction enzyme, a nucleic acid polymer substrate, a tracer compatible with the restriction enzyme, and other components essential for the enzymatic reaction, enzymatically cleaving the nucleic acid polymer substrate to form a restriction product, analyzing the product of  
20 the cleavage reaction by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture. The density of the reaction mixture can also be at least about 0.01 g/cm  
25 greater than the liquid phase utilized in the chromatographic or electrophoretic protocol.

The present invention is also directed toward a method for a restriction enzyme reaction, the method comprising forming a reaction mixture comprising a  
30 restriction enzyme, a nucleic acid polymer substrate, a tracer compatible with the restriction enzyme, and other components essential for the enzymatic reaction, enzymatically cleaving the nucleic acid polymer substrate to form a restriction product, analyzing the product of  
35 the cleavage reaction by an electrophoretic protocol,

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wherein the tracer supplied to the reaction mixture is of adequate character and sufficient quantity to be visible during the electrophoretic protocol.

The present invention is further directed toward a method for forming an enzymatic composition, the method comprising combining a reaction component with a tracer, the reaction component being essential for an enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, the tracer being compatible with the enzyme, and the resulting composition having an optical density greater than about 15 at a visible wavelength of maximal tracer absorbance. A liquid which is compatible with the enzyme can also be added, wherein the liquid increases the density of the composition to at least about 1.1 g/cm<sup>3</sup>.

The present invention is still further directed toward a method for forming an enzymatic composition, the method comprising combining a reaction component with a tracer, the reaction component being essential for a polymerase reaction in which a nucleic acid product is polymerized from a complementary nucleic acid template, the tracer being compatible with the enzyme, and the resulting composition having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance. A liquid which is compatible with the enzyme can also be added, wherein the liquid increases the density of the composition to at least about 1.1 g/cm<sup>3</sup>.

Other features, objects and advantages of the present invention will be in part apparent to those skilled in the art and in part pointed out hereinafter. All references cited in the instant specification are incorporated by reference. Moreover, as the patent and non-patent literature relating to the subject matter disclosed and/or claimed herein is substantial, many relevant references are available to a skilled artisan

that will provide further instruction with respect to such subject matter.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention provides compositions comprising an essential component to an enzyme reaction and a tracer which can allow post-reaction analysis without further tracer addition. These compositions ("analysis reagent compositions") can also comprise a high density agent which can eliminate the need for  
10 further addition of a high density agent during post-reaction analysis. Methods of using these compositions, and methods of preparing them are also provided.

Prior art methods of using loading buffer components always added these components to the reaction mixture,  
15 either before or after the enzymatic reactions are executed. Such methods require an extra step to add the loading buffer components. An improvement over the prior art in the present invention is the provision of analysis reagent compositions comprising an essential component  
20 for an enzymatic reaction combined with the loading buffer components. As diagrammed in Figure 3, the approach of the present invention offers further efficiencies that were not achieved with prior art protocols. The loading buffer components are passively  
25 added with the essential component, thus eliminating the extra addition step required in the prior art to add the loading buffer. In laboratories where many enzyme reaction mixtures are prepared, the compositions and methods of the present invention can thus eliminate a  
30 considerable amount of work. These analysis reagent compositions are particularly useful for molecular biological enzyme reactions, particularly polymerase reactions and restriction enzyme reactions, since these

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reactions are often repetitively performed under standardized conditions.

The procedures disclosed herein which involve the molecular manipulation of nucleic acids are known to those skilled in the art. See generally Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press.

As used herein, the term "substrate" encompasses a component of an enzymatic reaction mixture which is a reactant in the reaction catalyzed by the enzyme. For example, in a restriction enzyme reaction, the substrate is the nucleic acid polymer which is cut by the enzyme. In a polymerase chain reaction, the substrates include the primer which is extended by the enzyme, and the nucleotides which are added to the growing nucleic acid polymer.

The present invention provides compositions comprising a reagent which is essential for an enzymatic reaction ("essential reagent") and a tracer which is compatible with the enzyme. A high density agent may also be included in these compositions. Methods are also provided wherein the compositions are used in an enzymatic reaction and the results are subsequently analyzed by electrophoresis or chromatography in a water soluble solvent.

The compositions and methods of the present invention are useful in conjunction with any enzymatic reaction where the reaction products are subsequently analyzed by a chromatographic or electrophoretic method. Enzymes which can be employed include those which modify or degrade proteins, lipids, carbohydrates, and metabolites, such as any kinase, protease, lipase, amylase, peroxidase, oxidase, oxygenase, and dehydrogenase. Enzymes which modify, cut, or synthesize nucleic acids are particularly suitable to be used with

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the present invention. Examples include any ligase, phosphodiesterase, DNase, exonuclease, RNase, phosphatase, kinase, terminal transferase, reverse transcriptase, restriction endonuclease, RNA polymerase, and DNA polymerase. Enzymes which are preferred for use with this invention are restriction endonucleases and DNA polymerases. More preferred are DNA polymerases; even more preferred are any thermostable DNA polymerase; most preferred is wild-type or modified Taq polymerase. These enzymes can be in any concentration which is useful for performing an enzymatic reaction. Preferred concentrations of Taq polymerase in the compositions of the present invention are 0.033 - 10 units/ $\mu$ l, more preferred concentrations are 0.06 - 5 units/ $\mu$ l, the most preferred concentration is 1 unit/ $\mu$ l. Preferred concentrations of restriction endonucleases in the compositions of the present invention are 0.1 - 1000 units/ $\mu$ l, more preferred concentrations are 1 - 100 units/ $\mu$ l, most preferred concentrations are 5 - 40 units/ $\mu$ l.

Tracers which can be used in this invention include detectable compounds which can be incorporated into the reaction mixture and not interfere significantly with the enzyme reaction. Such a tracer is designated herein as "compatible" with the enzyme. It is preferred that this compatibility be such that an enzyme composition with the tracer has at least 95% of the activity of the same composition without the tracer. More preferably, the tracer-enzyme composition has at least about 97% activity of the composition without the tracer, even more preferably at least about 99% activity, and most preferably about 100% activity. The tracer should also be stable enough in the tracer-enzyme composition to retain its compatibility with the enzyme even after a

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long storage period at an appropriate temperature, e.g. 1 year or more at -20°C.

The detectable signal imparted by the tracer can be visual, such as that imparted by a dye or fluorescent compound. The tracer can also impart a radioactive, electrochemical, spectrophotometric, or any other type of signal which can be detected sensually or with an instrument and which can serve as a useful marker in an analysis subsequent to the enzyme reaction. Preferred are tracers which impart a visual signal. The most preferred tracers are dyes which are colored under the conditions that the analysis is performed. Any color dye which is visible during the post-reaction analysis can be used; preferred are dyes which have a peak visible absorbance wavelength at between 430 and 617 nm; most preferable dyes have a peak visible absorbance wavelength at between 500 and 535 nm.

While any tracer compatible with the enzyme can be useful in the present invention, preferred tracers are highly soluble in the liquid phase of the post-reaction chromatographic or electrophoretic procedure. The tracer is preferably an anionic tracer. Particularly preferred tracers are anionic tracers such as salts of organic acid dyes or sulfonic acid dyes. The preferred salt counterion is an earth metal, most preferably  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ . Where the post-reaction analysis is an electrophoresis of a nucleic acid, preferred tracers are anionic dyes. Preferred concentrations of the tracer in an analysis reagent composition are concentrations for which the composition has an optical density (OD) of between about 5 and about 500; most preferred is about 300. In enzyme reaction mixtures prepared from the analysis tracer composition the preferred tracer concentration has an OD of between 1 and 100; more preferred is 15 to 50; most preferred is 15.

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Commercial preparations of tracers are often inhibitory to enzyme activity when used in the concentrations recited above. This inhibition can often be overcome by further purification of the tracer, for example by reverse phase desalting, recrystallization, acid precipitation, or chromatographic methods such as reverse phase, normal phase, or ion exchange chromatography. Where the tracer is an anion, such as disclosed in Example 1 below, enzyme inhibition can also be overcome by replacing the counterion with an alkaline earth-metal. Preferred alkaline earth-metals for this purpose are  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ; most preferred is  $\text{Mg}^{++}$ .

High density agents useful for the present invention include any solute in which the tracer is soluble and which is compatible with the enzyme when diluted in the final reaction mixture, and which is dense enough to assist in the addition of reaction mixture to the analytical process. To provide such assistance, the density of the reaction mixture should be at least about 0.01 g/cm<sup>3</sup> greater than the density of the liquid phase of the analytical process (e.g. the electrophoresis or chromatographic buffer). A somewhat higher density (for example, about 0.05 g/cm<sup>3</sup> greater than the analytical liquid phase) would provide greater assistance in the addition of the reaction mixture, and is thus more preferred. These densities may be provided using the preferred density of an essential enzyme component/tracer/high density agent composition of about 1.14 g/cm<sup>3</sup>. Higher densities are also useful, however, provided they are compatible with the enzyme at the concentration used in the enzyme reaction.

Examples of solutes which are generally compatible with enzymes at the concentrations required to provide sufficient density are sucrose or other sugars, glycerol, and betaine (trimethylglycine). Glycerol is preferred.

Glycerol at a concentration of 1.5% in water is about 0.01 g/cm<sup>3</sup> more dense than water, and would thus provide assistance in applying a sample to an analytical process where water is the liquid phase. Glycerol at a  
5 concentration of 50% in water has a density of about 1.14 g/cm<sup>3</sup>. Thus, 50% glycerol is preferred as the high density agent in an essential enzyme component/tracer/high density agent composition.

A scheme which is useful for evaluating the  
10 compatibility and effectiveness of a tracer and/or high density agent ("loading buffer component") for this invention is shown in Fig. 4. In this scheme, the desired physical characteristics of the loading buffer component is determined. For example, the desired color  
15 and charge of a dye to be used as a tracer is decided. Next, a set of candidates (e.g. red, anionic dyes) is assembled for testing to evaluate other desired properties (e.g. enzyme compatibility, lack of interference in genetic transformation protocols, etc).  
20 The candidates are then tested for these desired properties, preferably by performing the least laborious tests first, in order for the largest amount of undesirable candidates to be eliminated by the least amount of screening. When desirable loading buffer  
25 components are selected, the formulation of loading buffer component and essential reagent is prepared, and its effect on the enzyme reaction and subsequent analysis is characterized.

Any essential reagent can be selected to be combined  
30 with the loading buffer component to formulate a composition of the present invention. The selection of an essential reagent for this purpose can depend on factors such as:

- whether it is desired to be able to determine if the  
35 essential reagent has been added to the enzyme reaction

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mixture. For example, if the essential reagent is an enzyme and the loading buffer component is a dye, then one can easily determine if the enzyme has been added to the reaction mixture by determining if the reaction mixture is colored.

5       - whether the essential reagent might be added at varying concentrations in several reaction mixtures, or whether there are alternative formulations of essential reagents which might be added. For example, a particular

10       buffer solution can be used at different concentrations by several different restriction endonucleases. See Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press, at pp. 5.28-5.31. The concentrated

15       buffer solution may not be a preferred essential reagent to combine with the loading buffer components under those conditions, since the final reaction mixtures could have varying concentrations of the loading buffer components, depending on the enzyme used. However, where the

20       concentrated buffer solution is generally always diluted a particular amount, such as with 10X buffers which are often provided for particular enzymes (e.g. restriction enzymes), the loading buffer components can be usefully provided in combination with these solutions.

25       Examples of essential reagents which can be combined with loading buffer components to formulate a composition of the present invention are: enzyme, concentrated enzyme buffer (e.g. 10X buffer), a nucleotide or primer reagent in the case of DNA or RNA polymerases, or a coenzyme such

30       as NADPH or ATP. The preferred essential agent for this purpose is the enzyme, since it is often desirable to be able to ascertain if enzyme addition has taken place, and since enzyme concentrations in reaction mixtures are generally not widely varied. A colored enzyme

35       formulation also has the advantage of allowing one to



determine if complete mixing of the enzyme has taken place. If the solution is uniformly colored then the enzyme is uniformly distributed. Also, since a colored formulation is more readily visible than a clear  
5 formulation, a colored enzyme formulation also facilitates pipetting of the small volumes of enzyme which are often added to enzymatic reaction mixtures.

Inclusion of loading buffer components with the substrate is usually not preferred because the substrate  
10 composition and concentration often varies between individual enzyme reactions. For a PCR reaction, however, the loading buffer components can be advantageously added with the nucleotide substrates, since the concentration of these reagents generally do  
15 not vary between individual PCR reactions.

As contemplated by the present invention, the analysis of the product of an enzyme reaction can be by any method which is suitable for the product in question. Chromatographic and electrophoretic methods are  
20 particularly suitable. Suitable chromatographic methods include liquid chromatography ("LC"), particularly gel permeation chromatography. In LC, a high density agent would facilitate the loading of the reaction mixture containing the product onto a chromatographic column, and  
25 a visible tracer would allow one to follow the progression of the sample through the column.

For applications where the product to be analyzed is a nucleic acid polymer, electrophoretic methods are preferred. In this regard, the compositions and methods  
30 of the present invention are useful for agarose gel electrophoresis (e.g. to analyze products of PCR, restriction endonuclease digestion, ligation reactions, etc), and polyacrylamide gel electrophoresis (e.g. analysis of sequencing reactions). Polyacrylamide gel  
35 electrophoresis is also facilitated by the present

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invention when used to analyze protein products of enzymatic reactions.

The following examples illustrate the invention, but are not to be taken as limiting the various aspects of the invention so illustrated.

**Example 1. Identification and formulation of a Taq DNA polymerase with tracers and high density reagent**

A composition conforming to the present invention was developed to facilitate analysis of products resulting from PCR. The composition comprises an essential PCR component, Taq DNA polymerase, with sufficient glycerol to facilitate the application of the reaction product to an agarose gel for electrophoretic analysis. The composition also comprises a red dye that aids in visualization and mixing of the enzyme in the reaction mixture. The red dye also serves as a tracer to follow the progressive movement of PCR products through an agarose gel during electrophoresis. The color red was selected for aesthetic reasons and confers no particular advantage as a tracer.

Fig. 5 summarizes the steps taken to develop this composition. Since nucleic acid products of PCR are highly anionic, they are applied to the agarose gel near the anode and move toward the cathode as the electrophoresis progresses. Therefore, to be useful as a tracer in electrophoresis the dye molecule is preferably anionic. Figure 6 summarizes the selection process. From 180+ red dyes (absorbance max between 450 and 570 nm) (Table 1) approximately 40 anionic dyes were selected (Table 2).

Table 1  
Dyes initially considered

	Dye	$\lambda_{\max}$
	Bis-N-methylacridinium nitrate	430
5	4-(p-Nitrophenylazo)-resorcinol	432
	Auramine O	432
	Martius Yellow	432
	3',3'',5',5''-Tetraiodophenolsulfonephthalein	433
	6'-Butoxy-2,6-diamino-3,3'-azodipyridine	435
10	Quinoline Yellow A, spirit soluble	435
	m-Cresol Purple, sodium salt	436
	Methyl Red, sodium salt	437
	Methylthymol Blue, water soluble	438
	a-Naphthyl Red	439
15	Palatine Fast Yellow BLN	440
	Twort Stáin	440
	Pyrocatechol Violet	441
	Acridine Yellow G	442
	Mordant Brown 33	442
20	2-(5-Bromo-2-pyridylazo)-5-(dimethylamino)phenol	443
	Disperse Orange 3	443
	Acid Yellow 99	445
	Thymolphthalein monophosphoric acid, disodium salt hydrate	445
	Acid Orange 51	446
25	Eriochrome Cyanine R	446
	Malachite Green Carbinol base	446
	Ethyl Red	447
	Chrysoidin	449
	Orange G	475
30	Sudan I	476
	trans-p-Carotene	478
	Fast Yellow	480
	Pyrogallol Red	480
	Direct Black 22	481
35	Crocein Orange G	482
	Rosolic Acid	482
	Disperse Orange 1	483
	Eriochrome Red B	483
	Orange 11	483
40	Thorin I	483
	Purpurin	485
	Quinizarin	485
	Mordant Brown 1	487
	Acridine Orange	488
45	Para Red	488
	Acridine Orange	489

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	Dye	$\lambda_{\max}$
	Acid Orange 8	490
	Astrazon Orange G	490
	Fluorescein diacetate	490
	Fluorescein isothiocyanate, isomer I	490
5	Quinalizarin	490
	Tropaeolin 0	490
	Zincon	490
	Zincon, monosodium salt	491
	Fluorescein, water soluble	492
10	Acridine Orange hydrochloride	492
	Mordant Brown 48	493
	Methyl Red hydrochloride	493
	Sudan 11	494
	Acid Red 183	494
15	Reactive Orange 16	495
	Carminic acid	495
	Disperse Red 19	495
	Fluoresceinamine, isomer 11	496
	Fluorescein	496
20	Fluoresceinamine, isomer I	497
	Brilliant Yellow	497
	Congo Red	498
	Acid Red 97	498
	Cochineal	499
25	Arsenazo I	499
	Fluorexon	500
	Benzopurpurin 4B	500
	Mordant Brown 4	500
	Reactive Red 8	501
30	Acid Alizarin Violet N	501
	Rhodamine 123 dehydrate	502
	Darrow Red	503
	Disperse Red 1	503
	Xylidine Ponceau 3RS	505
35	Acid Red 106	505
	Acid Red 88	505
	Biebrich Scarlet, water soluble	505
	Nuclear Fast Red	506
	Acid Red 4	506
40	New Coccine	507
	2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene-disulfonic	507
	Direct Red 23	507
	Merbromin	507
	Methyl Orange	507
45	Sudan III	507
	Toluidine Red	507

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	Dye	$\lambda_{\max}$
	Acid Red 4	508
	Acid Red 8	508
	Direct Red 81	508
	2',7'-Dichlorofluorescein	509
5	Brilliant Crocein MOO	510
	Chromotrope 2R	510
	Basic Red 29	511
	Acid Red 151	512
	Chromoxane Cyanine R	512
10	Quinalizarin	512
	Acid Red 37	513
	Acid Red 114	514
	Chromotrope 2B	514
	Eosin B	514
15	Eosin Y	514
	Ponceau SS	514
	Acid Red 150	515
	Chromotrope FB	515
	Acid Red 40	516
20	Azocarmine B	516
	Mordant Blue 9	516
	Reactive Red 4	516
	Cibacron Brilliant Red 3B-A	517
	Disperse Red 13	517
25	Eosin Bluish blend	517
	4,5,6,7-Tetrachlorofluorescein	518
	Bordeaux R	518
	Oil Red 0	518
	Acid Violet 7	520
30	Methyl eosin	520
	Ponceau S	520
	Rose Bengal, bis(triethylammonium) salt	520
	Sudan IV	520
	Amaranth	521
35	Emodin	521
	Eosin Y, free acid	521
	Giemsa Stain	521
	Oil Red EGN	521
	Purpuri'n	521
40	Azure A eosinate	522
	Diiodofluorescein	522
	Direct Red 75	522
	Eosin B, spirit soluble	522
	Jenner Stain	522
45	Leishman Stain	522
	May-Grbnwald Stain	522
	Wright Stain	522

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	Dye	$\lambda_{\max}$
	Wright Stain, solution in methanol	522
	Azure B eosinate	523
	Zincon, monosodium, salt	523
	Acid Blue 120	524
5	Azure 11 eosinate	524
	Eosin Y lactone	524
	Rhodamine 6G	524
	Tetrachrome Stain (MacNeal)	524
	Erythrosin B	525
10	Erythrosin Yellowish blend	525
	Ethidium bromide	525
	Acid Violet 5	527
	Plasmocorinth B	527
	Eriochrome Blue Black 2B	528
15	Quinaldine Red	528
	Rhodamine 6G Perchlorate	528
	Rhodamine 6G tetrafluoroborate	529
	Sulforhodamine G	529
	Violamine R	530
20	Chromotrope 2R	530
	Safranin O (Y,T)	531
	Alum Carmine	531
	Carmine	532
	Acid Red 1	532
25	Acid Red 106	532
	Ethyl Eosin	533
	Arsenazo 111, sodium salt hydrate	533
	Erythrosin B, spirit soluble	533
	Sudan Red 7B	534
30	Ruthenium Red	535
	Nuclear Fast Red	538
	Acid Red 40	540
	Alizarin Violet 3R	540
	Neutral Red	542
35	Aluminon	543
	Rhodamine B	544
	Basic Fuchsin	544
	Basic Fuchsin, special for flagella	544
	Pararosaniline base	544
40	Rhodamine B base	545
	Acid Fuchsin, calcium salt	545
	Acid Violet 17	545
	Aurintricarboxylic acid	545
	Aurintricarboxylic acid, trisodium salt	545
45	Pararosaniline acetate	546
	Acid Fuchsin, sodium salt	547
	Carbol Fuchsin	547

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	Dye	$\lambda_{\max}$
	Alizarin Blue Black B	548
	Phloxine B	548
	Pyronin Y	548
	Rose Bengal	549
5	Basic Fuchsin, biological stain	549
	Direct Violet 51	552
	9-Phenyl-2,3,7-trihydroxy-6-fluorone	552
	Bromopyrogallol Red	552
	Phenolphthalein	552
10	Rhodanile Blue	553
	New Fuchsin	553
	Nile Red	553
	Pyronin B	554
	Sulforhodamine B	556
15	Alizarin Red S monohydrate	557
	Methylene Violet 3RAX	557
	PhenolRed	559
	Rose Bengal, bis(triethylammonium) salt	560
	Arsenazo III	560
20	Pinacyanol chloride	563
	Acid Blue 161	563
	Carmin	565
	Nigrosin, alcohol soluble	566
	Acid Blue 113	566
25	o-Cresolphthalein	567
	Alizarin	567
	Sulfonazo 111, tetrasodium salt	569
	Palatine Chromé Black 6BN	570
	Brilliant Black BN	570
30	Cresol Red	617
	Bromocresol Green (also broad absorbance at 417 nm)	617

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Table 2  
Properties of anionic dyes

No	Dye	$\lambda_{\max}$ (nm)	Color	EtOH	Qiagen	PCR
5	1 Orange G	475	X			
	2 Acid Red 150	515		X		
	3 Acid Red 88	505	solubility			
	4 Acid Red 106	532			X	
	5 m-Cresol Purple, sodium salt	436	X			
	6 2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene-disulfonic acid, trisodium salt	507			X	
10	7 Mordant Blue 9	516	X			X
	8 Chromotrope 2R	510				X
	9 Pyrogallol Red	480				X
	10 Reactive Red 4	516				
	11 Disperse Orange 1	483	X			
15	12 Congo Red	497	X			
	13 Direct Red 81	508		X		
	14 Phloxine B	548	X			
	15 Eriochrome Cyanine R	446	X			
	16 Acid Violet 17	545	X			
	17 Chromotrope 2B	514			X	
20	18 Zincon, monosodium salt	490	X			
	19 Methyl Red, sodium salt	437	X			
	20 Acid Orange 8	490	X			
	21 Rosolic Acid	482	solubility			
	22 Eosin Y	514	X			
	23 Bordeaux R	518				X
25	24 Acid Red 106	505				
	25 Acid Red 4	506				
	26 Acid Red 1	532				
	27 Bromocresol Green (also broad absorbance at 417 nm)	617	X			
	28 Ponceau S	520		X		
	29 Benzopurpurin 4B	500	solubility			
30	30 Acid Orange 51	446	X			
	31 Amaranth	521				
	32 4-(p-Nitrophenylazo)-resorcinol	432	X			
	33 Biebrich Scarlet, water soluble	505		X		
	34 Martius Yellow	432	X			
	35 Reactive Orange 16	494				X
35	36 Direct Violet 51	549	X			
	37 Chromotrope FB	515			X	
	38 Direct Red 75	522		X		
	39 Acid Violet 5	527				
	40 Acid Red 97	498				X

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Each of these dyes were dissolved in water and those that were not particularly red (i.e. were too yellow/orange or purple), or lacked sufficient solubility, were removed from consideration (Table 2, those marked under "Color").

- 5        The 20 dyes which remained were assayed for their ability to be removed from DNA by ethanol precipitation. To 1  $\mu$ g of lambda DNA enough dye was added to yield a highly colored solution. Addition of two volumes of 3M ammonium acetate, then 6 volumes of ethanol followed.
- 10      The DNA was pelleted by centrifugation. The appearance of a colored pellet caused the dye to be removed from consideration (Figure 6, EtOH ppt.; Table 2, those marked under "EtOH"). In the presence of the remaining dyes, one  $\mu$ g quantities of DNA were purified by solid phase
- 15      extraction on Qiagen PCR product purification columns (Qiagen, Hilden, Germany) according to the manufacturers protocol. Dyes that yielded colored eluants were dropped from consideration (Figure 6, labeled "Qiagen", Table 2, those marked under column labeled "Qiagen"). The assays
- 20      performed to this point were done at the onset of the screening because they were the least laborious.

         The dyes that survived these preceding tests were included in a PCR toxicity study. The dyes were added to PCR reactions at concentrations considered adequate for

25      product performance. Enough of a 2X PCR master mix consisting of Taq DNA polymerase, 0.1 u/ $\mu$ l (Sigma Chemical Co., St. Louis, MO), PCR buffer (2X) (Sigma), dNTP's (200  $\mu$ M each) (Sigma),  $\alpha^{32}$ PdCTP (Amersham USA, Piscataway, NJ), target DNA (lambda, 2 ng/ $\mu$ l) (Sigma) and

30      primers (Perkin-Elmer 500 bp control, 2  $\mu$ M) (Perkin-Elmer, Norwalk, CT) was prepared to accommodate all 11 remaining dyes plus three no dye controls. Ten  $\mu$ l of the 2X master mix was dispensed into reaction tubes followed by addition of 10  $\mu$ l of aqueous darkly colored dye

35      solutions or water (controls). The PCR cycling protocol

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was 20 cycles of 94/55/72°C at one minute each. 20 µl of quench solution (50 µg/µl calf thymus DNA, 20 mM EDTA) was added followed by precipitation with 40 µl 40% trichloroacetic acid (TCA)/4% sodium pyrophosphate (NaPPi). The reactions were filtered on glass fiber filters, washed with 5% TCA/2% NaPPi and counted by scintillation methods. This quench/precipitation procedure will henceforth be referred to as "TCA precipitation". As shown in Figure 7, some dyes significantly inhibited the PCR reaction such that little or no product resulted (i.e. numbers 8, 9, 10, 35 and 40). However, other dyes were relatively inert (i.e. 23, 24, 25, 26, 31, 39). The dyes that inhibited PCR were dropped from consideration (Fig.6, PCR Tox, Table 2, those marked under "PCR"). Number 23 was dropped because its per cycle yield was substantially lower than the other dyes (Fig.7). Per cycle yield was calculated assuming the overall yield was the per cycle yield raised to the 20<sup>th</sup> power (Figure 7).

The dyes which remained after the PCR toxicity screen were further screened for their toxicity on ligation and transformation, two downstream procedures often carried out using unpurified PCR products. As shown in Figure 8, ligation in the presence of the remaining dyes (24, 25, 26, 31 and 39 lanes 3-7 respectively) was equivalent to the no dye control (lane 2). Lane 1 is a control which had no ligase. Ligations were carried out as described in the figure legend. The same dyes were also tested for suitability in a ligation/transformation protocol. Transformation efficiency was not compromised by the presence of dye (Figure 9). Increased efficiencies, as evidenced in the figure, were not investigated. The experiment was performed as summarized in the figure legend.

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On the basis of performance to this point, the remaining dyes were considered substantially equivalent as tracer candidates. Acid violet 5 (number 39, Table 2) was chosen as a possible finalist candidate. Using this dye in a reaction mix at an absorbance of 10 (at its absorbance maximum of 527 nm) a PCR reaction was performed. The inclusion of this dye in a reaction mix resulted in a minimum of 50% loss (relative to the same reaction without the dye) in PCR product yield (Figure 10) as measured by TCA precipitation. This was considered unacceptable, but was overcome by further development efforts.

In general, the available dyes, as well as those investigated, are derived from unrelated applications. That is, many are used as fabric dyes, food colorings, biological stains etc. As such, they are available in various states of purity. In the case of the finalists, they all had purities of 80% or less, as determined by the product labels. In further experiments, PCR yields (determined by TCA precipitation) in reactions containing various concentrations of acid violet 5 (determined by  $A_{527}$ ) were compared before and after purification of the crude dye by reverse phase desalting. PCR product yield was much less susceptible to dye concentration for the desalted compound relative to the crude compound (Figure 11). Since reversed phase desalting is not an attractive method for large scale dye purification, acid precipitation followed by ammonium hydroxide dissolution/evaporation to produce the ammonium dye was investigated. This procedure should result in an essentially salt free product. These procedures (reversed phase desalting and acid/ammonium hydroxide) were carried out for acid red 1 (No. 26, Table 2) and acid violet 5 (No. 39). It was found that the ammonium dyes were more PCR toxic than the sodium (i.e. desalted) dyes (Figure 12a). The dyes were

converted to their magnesium salts to further  
characterize the effect of counterion identity. This was  
accomplished by addition of magnesium chloride (excess)  
to a solution of the crude dye. The magnesium dye that  
5 immediately precipitated was recrystallized from water.  
As shown in Figure 12a, the Mg salts of both acid red 1  
and acid violet 5 was much less toxic to PCR than either  
the sodium or ammonium salts. In analogy with the  
magnesium salts, the calcium and zinc salts of acid red 1  
10 were prepared to investigate whether the effect was  
divalent vs. monovalent cation or cation identity  
specific. Figure 12b demonstrates that the magnesium  
salt was least toxic. From these data it was concluded  
that the dye was likely sequestering magnesium from the  
15 PCR reaction which caused decreased product yields.

A dye formulation of the magnesium salts of 80% acid  
red 1 / 20% acid violet 5 was created (percentages based  
on absorbance at the wavelength of each dye's maximum  
absorbance [acid red 1 = 531 nm, acid violet 5 = 528  
20 nm]). That particular ratio was used for aesthetic  
reasons, however any ratio, or either of the dyes  
individually would be similarly effective. To further  
investigate the effects of  $Mg^{++}$ , since the dye is  
supplying magnesium to a magnesium dependent reaction  
25 (i.e. PCR), free  $Mg^{+2}$  concentration contributed by the dye  
to the reaction was determined. This was determined by  
varying the magnesium concentration in dye containing vs.  
dye free reactions in a dose response manner. Figure 13  
shows that for products ranging from 500 to 3000 bp, the  
30 difference between red and white Taq at the midpoint of  
the magnesium concentration titrations is approximately  
0.4 mM (0.37 +/- 0.04). The 10X buffer usually supplied  
with Taq was reformulated to account for this  
perturbation (i.e. the concentration of  $MgCl_2$  was changed  
35 from 15 to 11 mM in the 10X buffer).

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A preferred composition was formulated at 1 u/ $\mu$ l Taq polymerase in Taq storage buffer (consisting of 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal® CA-630, 50% glycerol in water) with the magnesium formulation of dye at a total absorbance of 300. The dye composition was 80% acid red 1, 20% acid violet 5 (100% = absorbance of acid red 1 at  $\lambda_{\max}$  + absorbance of acid violet 5 at  $\lambda_{\max}$ , absorbance of acid red 1 = 240, acid violet 5 = 60). This formulation is designated "REDTaq™". When added to a PCR reaction mixture at 0.05 u/ $\mu$ l Taq, the total dye absorbance is 15. The dye combination at this concentration was visible in a subsequent agarose gel electrophoresis of the completed reaction mix, yet the combination was relatively non-toxic to PCR. A lower concentration of the dye in the reaction mixture would be difficult to see during a subsequent agarose gel electrophoresis. As a comparison, the previously discussed prior art Taq-dye formulation, Red Hot DNA Polymerase, has an absorbance of 3.3 at 572 nm, and 4.6 at 435 nm. At the recommended concentration in a PCR reaction mixture, Red Hot DNA Polymerase has an absorbance of 0.033 and 0.046, at 572 and 435 nm, respectively. Therefore, in contrast to REDTaq™, the Red Hot DNA Polymerase formulation would not be useful as a tracer in an electrophoretic analysis of a PCR reaction.

PCR products prepared using the REDTaq™ formulation with the 10X buffer described above (with 11 mM MgCl<sub>2</sub>) were compared with conventional Taq/10X buffer (with 15 mM MgCl<sub>2</sub>). Figure 14 shows a 1% agarose gel of amplification products resulting from this comparison. From the gel it is apparent that the amplifications using REDTaq™ were equivalent to those using Taq without dye. The exception to this is amplification of the 3 kb fragment, where the amplification with conventional Taq failed for unknown reasons (Lane 4). However, when

product yields were compared for a variety of target sizes (Figure 15), both conventional Taq and REDTaq™ did effectively amplify a 3 kb target. In that comparison, product yield was not compromised by REDTaq™. When a similar comparison was made with RediLoad, a commercial formulation of a red loading buffer (without an essential reaction component) which is added before a PCR reaction, the Rediload product reduced PCR product yield by approximately 10% (Figure 16), relative to the same reaction without Rediload.

**Example 2. Determination of the compatibility of a dye with restriction endonucleases**

The effectiveness of restriction enzymes in cutting target DNA when a dye is present in the reaction mixture was evaluated. A variety of restriction enzymes were assayed for the detrimental effect of adding Amaranth (No. 31, Table 2) to a restriction digest as assayed by agarose gel electrophoresis. Nde I-cut pUC19 plasmid was prepared. This linearized plasmid was then digested with one of several restriction enzymes which normally cut pUC19 at a polylinker site. Thus, each enzyme would be expected to yield a product of similar sizes (ranging from 212 to 263 bp). The restriction enzyme digests were performed in the presence, or absence, of Amaranth dye, and at various concentrations of the enzyme. The results of this experiment are shown in Fig. 17. Column 1 (the leftmost column) contains 100 bp molecular weight ladders. Columns 2-4 contains cleavage products from sequential 5-fold dilutions of the restriction enzymes in the presence of dye. Columns 5-7 are as columns 2-4 but without dye. Columns 2 and 5 contained the restriction enzyme at 1/10<sup>th</sup> the suppliers concentration (i.e. the enzyme was considered a 10X concentration). The buffers used for the digests were as recommended by the supplier.

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The enzymes used are listed along the right side of the gel. The relative susceptibility of the enzyme to the dyes' presence is reported next to the enzyme. The sizes of the small bands in the electrophoresis runs range from 212 to 263 bp. The larger, brighter bands contain the full length NdeI-cut pUC19 (2686 bp) and/or the larger fragment of the NdeI-cut pUC19 which was also cut with the test enzyme (2423-2474 bp). +++ equals inert, blank is completely toxic. This preliminary experiment reveals that the restriction enzymes tested were relatively insensitive to dye addition. That is, with the exception of KpnI, Amaranth was relatively inert in these reactions. This experiment was conducted using commercially prepared (crude) dye (dye content approximately 90%). Based on the previously discussed results with Taq polymerase, a dye screening and possible cleanup/counterion exchange, similar to that used in Example 1, should result in the discovery of a dye system that would work for a majority of restriction enzymes.

To enable direct gel loading, the restriction digest would have to contain a component that made the solution more dense than the electrophoresis buffer. Like the PCR product, glycerol or another high density agent should suffice. Generally, star activity (alternate site cutting of DNA due to high glycerol content) can occur at glycerol concentrations above 5%. For REDTaq™, solutions as low as 1.5% glycerol were effective as a high density agent. Thus, it is clearly possible that restriction digest reagents could be formulated to contain enough glycerol or other density increasing solutes to allow for direct gel loading.

Other features, objects and advantages of the present invention will be apparent to those skilled in the art. The explanations and illustrations presented herein are intended to acquaint others skilled in the art

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with the invention, its principles, and its practical application. Those skilled in the art may adapt and apply the invention in its numerous forms, as may be best suited to the requirements of a particular use.

- 5 Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention.

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